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ORIGINAL ARTICLE

Lipopolysaccharide inhibits hepatic gluconeogenesis in rats: The role of immune cells

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ABSTRACT

Aims/Introduction: Bacterial septicemia has diverse clinical symptoms including severe hypoglycemia. However, sepsis-induced hypoglycemia has not yet been examined in detail. The aim of the present study was to investigate the mechanisms underlying hypoglycemia in sepsis.

Materials and Methods: We induced endotoxin shock in rats using lipopolysaccharide (LPS). After an intraperitoneal injection of LPS, we measured gluconeogenesis using the pyruvate tolerance test. The effects of LPS on glucose metabolism were investigated in perfused livers and isolated hepatocytes. Furthermore, its effects on the production of inflammatory cytokines were examined in isolated splenocytes. The interaction between splenocytes and hepatocytes in response to LPS was investigated *in vitro* using a co-culture of splenocytes and hepatocytes.

Results: In the pyruvate tolerance test, the pretreatment with LPS decreased gluconeogenesis. The *in vivo* pretreatment of rats with LPS did not inhibit glucose production in perfused livers. The *in vitro* treatment of isolated hepatocytes with LPS did not decrease hepatic gluconeogenesis. Although LPS increased the production of inflammatory cytokines (tumor necrosis factor-α, interferon-γ, interleukin-1β, interleukin-6 and interleukin-10) and nitric oxide in isolated splenocytes, only nitric oxide significantly inhibited gluconeogenesis in isolated hepatocytes. When splenocytes and hepatocytes were co-cultured in medium containing LPS, the messenger ribonucleic acid expression of glucose-6-phosphatase in hepatocytes was suppressed.

Conclusions: LPS reduced hepatic gluconeogenesis, at least in part, by stimulating the production of nitric oxide in splenocytes. This effect could contribute to the mechanisms responsible for septicemia-induced hypoglycemia.

INTRODUCTION

Sepsis is a systemic response caused by pathogenic microorganisms, and is accompanied by a number of clinical symptoms[1]. These symptoms include endotoxin shock caused by lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. Septicemia causes not only hyperglycemia, but also hypoglycemia. Although the incidence of hypoglycemia is markedly lower than that of hyperglycemia, the mechanisms underlying hypoglycemia in sepsis must be considered. Endotoxic hypoglycemia plays an important role in predicting the severity and prognosis of sepsis[2].

Several studies examined the mechanisms responsible for the development of hypoglycemia in animals or humans with sepsis. LPS was shown to increase insulin sensitivity[3], and inhibit hepatic gluconeogenesis[4,5]. To the best of our knowledge, the mechanism underlying hypoglycemia in sepsis has not yet been elucidated. LPS stimulates immune cells and increases the secretion of inflammatory cytokines, such as tumor necrosis factor-α

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(TNF-α). It is also a representative ligand of Toll-like receptor 4, which is predominantly expressed in macrophages and dendritic cells[6]. Toll-like receptor 4 is crucially involved in immune responses, which are the first line of defense against infectious diseases[7].

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme that catalyzes the rate-limiting step of gluconeogenesis, and glucagon increases its activity[8]. TNF-α, a protein induced by LPS, inhibits glucagon-stimulated PEPCK gene expression and gluconeogenesis[9,10]. LPS also increases hepatic insulin sensitivity[3], and accordingly, might exert hypoglycemic effects. In contrast, Sugita *et al*[11] previously reported that hyperglycemia and insulin resistance were induced by LPS. Thus, glucose metabolism during septic shock has not yet been examined in detail.

Macrophages activated by LPS generate nitric oxide (NO), a highly reactive molecule with a number of important biological actions. NO is synthesized by three different NO synthase (NOS) isoforms: inducible NOS (iNOS), endothelial NOS and neuronal NOS. iNOS is primarily expressed in immunocytes, and produces large quantities of NO when stimulated by LPS, leading to marked increases in plasma NO concentrations. Horton *et al.*[12] showed that the addition of NO donors to incubation medium inhibited gluconeogenesis in isolated hepatocytes, which supports increases in plasma NO levels *in vivo* also inhibiting hepatic gluconeogenesis.

We herein investigated the mechanisms underlying glucose dyshomeostasis in sepsis, and clarified whether LPS inhibits hepatic gluconeogenesis. Our results showed that LPS failed to exert any direct effects on gluconeogenesis in isolated hepatocytes or perfused livers. Furthermore, using isolated splenocytes, we measured the production of representative inflammatory cytokines (TNF-α, interferon [IFN]-γ, interleukin [IL]-1β, IL-6 and IL-10) and NO after stimulation with LPS. Our results showed that LPS increased the secretion of TNF-α, IFN-γ, IL-1β, IL-10 and NO from splenocytes. We measured glucose production in isolated hepatocytes after stimulations with TNF-α, IFN-γ, IL-1β, IL-6, IL-10 and NO, and found that gluconeogenesis in primary hepatocytes was suppressed by the addition of sodium nitroprusside (SNP), a NO donor, but not by TNF-α, IFN-γ, IL-1β, IL-6 or IL-10. Our results showed that LPS suppressed the messenger ribonucleic acid (mRNA) expression levels of G6Pase, a master regulator of glucose production, in hepatocytes co-cultured with splenocytes. Therefore, LPS might inhibit hepatic gluconeogenesis, at least partly, by increasing the release of NO from the spleen, and does not appear to act directly on the liver.

METHODS

Animals

Male Wistar rats, weighing approximately 180 g (8 weeks-of-age), were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and maintained in a temperature-controlled (25 ± 1°C) environment with a 12-h light/dark cycle. Rats had free access to standard laboratory chow and water, and were maintained and used in accordance with the Guidelines for Animal Experiments of Tezukayama Gakuin University.

Effects of LPS on blood glucose

Blood glucose levels were measured, followed by an intraperitoneal injection of saline (control) or LPS from *Escherichia coli* serotype O111:B4 (0.1–0.5 mg/kg; Sigma Aldrich, St. Louis, MO, USA). Blood glucose levels were measured 2, 4, 6 and 8 h after the LPS injection using the glucose oxidation method (glucose CII test; Wako, Osaka, Japan).

Pyruvate tolerance test

LPS from 0.1–0.5 mg/kg or saline (control) was given in a single intraperitoneal injection to 16-h fasted rats weighing approximately 250 g. After 4 h, glucose levels were measured in blood collected from the tail vein, and pyruvate (1 g/kg) was then injected intraperitoneally into rats. Blood was serially collected at 30-min intervals after the pyruvate injection. In NOS inhibitor pretreatment experiments, rats were injected intravenously with N^G-monomethyl-L-arginine (L-NMMA; 10 mg/kg) 20 min before the intraperitoneal injection of LPS (0.5 mg/kg).

Preparation and incubation of hepatocytes and splenocytes

Hepatocytes were isolated from male Wistar rats fasted for 16 h by collagenase digestion as described previously[13]. Isolated hepatocytes (>90% viable by the trypan blue exclusion test) were seeded on 24-well plastic plates (Corning, New York, NY, USA) at a density of 5.0×10^5 cells per well. Cells were cultured in Krebs–Ringer buffer and incubated in 95% O_2/5% CO_2 at 37°C for 2 h in the presence of 0.24 mmol/L 3-isobutyl-1-methylxanthine and gluconeogenic precursors (10 mmol/L lactate and 1 mmol/L pyruvate) with/without LPS (0.02–20.0 μg/mL). After the incubation, medium was centrifuged at 50 *g* for 3 min to separate supernatants and pellets.

Splenocytes were harvested from the spleens of rats by homogenization. Crude cells were left to sink to the bottom of tubes for 1 min, and the supernatant was then centrifuged at 500 *g* for 3 min. Ammonium chloride was added to the precipitate in order to lyse red blood cells. Then, 30 s later, cells were washed twice with Dulbecco's Modified Eagle's Medium (DMEM). Isolated splenocytes were seeded on 10-cm plastic dishes at a density of 1.0×10^6 cells/mL, and cultured in DMEM supplemented with 100 μmol/L 2-mercaptoethanol with/without LPS (0.01–1.0 μg/mL).

Gluconeogenesis in perfused rat livers

Rat liver perfusion was carried out with the flow-through method, as described previously[14]. Details are shown in Appendix S1.

Effects of LPS on nitrate + nitrite, TNF-α, IFN-γ, IL-1β, IL-6 and IL-10 production in splenocytes

The concentrations of the LPS-induced cytokines, NO, IFN-γ, IL-1β, IL-6 and IL-10 in the culture medium of splenocytes

were measured after 4, 8, 16, and 24 h. NO is hydrolyzed in medium, and this is followed by the formation of NO stable metabolites (nitrate + nitrite [NOx]). NOx concentrations were measured using the Griess method (NO_2/NO_3 Assay Kit-CII; Dojindo, Kumamoto, Japan). Enzyme-linked immunosorbent assay was carried out to measure the levels of TNF-α (IBL, Gumma, Japan), IFN-γ (R&D systems, Minneapolis, MN, USA), IL-1β (R&D systems), IL-6 (Arigobio, Hsinchu, Taiwan) and IL-10 (R&D systems).

Effects of inflammatory cytokines on hepatic gluconeogenesis *in vitro*

Primary hepatocytes were prepared as described above, and cultured with SNP, TNF-α, IFN-γ, IL-1β, IL-6 and IL-10. SNP was used as a NO donor. After a 2-h incubation, the concentration of glucose in the supernatant was measured as described above.

Immunoblotting analysis

Western blotting was carried out as described previously with slight modifications[13]. Details are shown in Appendix S2.

Co-culture of splenocytes and hepatocytes

Isolated splenocytes were seeded on a polycarbonate membrane on the bottom of insert cups for 24-well plastic plates (Thermo Fisher Scientific, Roskilde, Denmark) at a density of 3.0×10^6 cells/cup, and were cultured for 24 h in DMEM supplemented with 100 μmol/L 2-mercaptoethanol with/without LPS (1.0 μg/mL). Freshly isolated hepatocytes were then co-cultured in 24-well plates with the above-described splenocytes for an additional 2 h in DMEM supplemented with/without LPS (1.0 μg/mL), without direct contact between splenocytes and hepatocytes.

Total RNA extraction, complementary deoxyribonucleic acid synthesis and real-time reverse transcription polymerase chain reaction

Total RNA was extracted from hepatocytes using Sepazol (Nacalai Tesque, Kyoto, Japan), and complementary deoxyribonucleic acid synthesis was synthesized using the PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan). SYBR Premix Ex Taq II (Takara Bio) was used for real-time polymerase chain reaction. The rat sequences for forward and reverse primers to detect PEPCK, G6Pase and β-actin as an inner control are shown in Table S1. Polymerase chain reaction was carried out with the Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s.

Effects of LPS on blood NOx concentrations

Blood NOx concentrations were measured, followed by an intraperitoneal injection of saline (control) or LPS (0.1–0.5 mg/kg). Blood was collected after 2, 4, 8 and 24 h, and the levels of NOx produced were measured as described above. In NOS inhibitor pretreatment experiments, rats were injected intravenously with L-NMMA (10 mg/kg) 220 min after the injection of LPS (0.5 mg/kg).

Statistical analysis

Comparisons between two groups were carried out using an unpaired Student's *t*-test. A one-way ANOVA followed by Dunnett's *post-hoc* test was carried out for more than two groups. A value of $P < 0.05$ was considered significant.

RESULTS

Time-course of effects of LPS on blood glucose levels

As the first step to elucidate the effects of LPS on glucose homeostasis, we examined the time-course of changes in blood glucose levels using rats. The intraperitoneal injection of LPS (0.1–0.5 mg/kg) or saline (control) did not significantly alter blood glucose levels (Figure 1).

Effects of LPS on the pyruvate tolerance test

We next evaluated the effects of LPS on hepatic gluconeogenesis using the pyruvate tolerance test. Four hours after the injection of LPS or saline intraperitoneally, elevations in blood glucose levels after the intraperitoneal administration of pyruvate were significantly suppressed in the LPS groups (Figure 2). This suppressive effect of LPS (0.5 mg/kg) on gluconeogenesis was significant 30 min (control, 6.3 ± 0.49 mmol/L; LPS, 4.6 ± 0.51 mmol/L, $P < 0.05$ vs control), 60 min (control, 6.1 ± 0.49; LPS, 3.6 ± 0.41 mmol/L, $P < 0.01$ vs control) and 120 min (control, 5.5 ± 0.15 mmol/L; LPS, 3.7 ± 0.42 mmol/L,

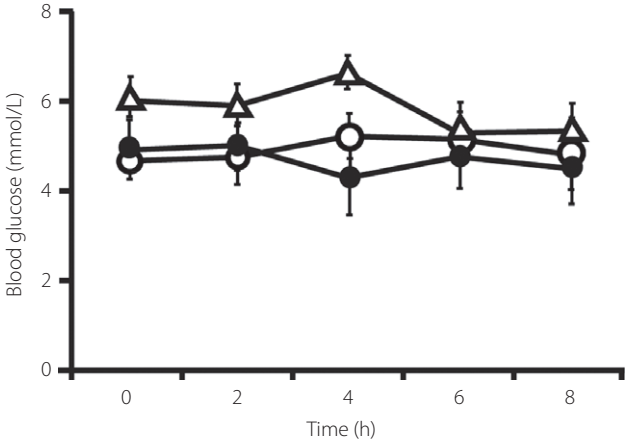

Figure 1 | No significant differences in blood glucose levels after an injection of lipopolysaccharide (LPS) or saline. Blood glucose levels of randomly fed Wistar rats were measured, followed by an injection of saline (control) or LPS (0.1–0.5 mg/kg bodyweight) intraperitoneally. Blood glucose levels were measured 2, 4, 6 and 8 h after the injection. Rats were provided with food *ad libitum*, even after the LPS injection. White circles, control; white triangles, LPS (0.1 mg/kg bodyweight); black circles, LPS (0.5 mg/kg bodyweight). Values are means ± standard error ($n = 5$).

$P < 0.01$ vs control) after the pyruvate injection. This suppressive effect of LPS (0.1 mg/kg) was significant 30 min (LPS, 4.2 ± 0.48 mmol/L, $P < 0.01$ vs control) and 120 min (LPS, 4.3 ± 0.47 mmol/L, $P < 0.05$ vs control) after the pyruvate injection. Blood glucose levels after the intraperitoneal injection of vehicle (saline), but without pyruvate injection were not significantly affected. These results show that LPS decreases gluconeogenesis from pyruvate.

Effects of LPS on gluconeogenesis in isolated hepatocytes

In order to investigate the direct effects of LPS on hepatocytes, we examined gluconeogenesis using isolated hepatocytes. The effects of LPS on glucose synthesis from 10 mmol/L lactate plus 1 mmol/L pyruvate in a 2-h incubation of hepatocytes with LPS are shown in Figure 3. LPS did not exert suppressive effects on hepatic gluconeogenesis in freshly isolated hepatocytes at concentrations ranging between 0.02 and 20.0 μg/mL. Glucose production by hepatocytes was significantly reduced by approximately 85% and 40% in the absence of gluconeogenesis precursors in incubation medium and in the presence of 5 mmol/L metformin, a representative inhibitor of gluconeogenesis, respectively.

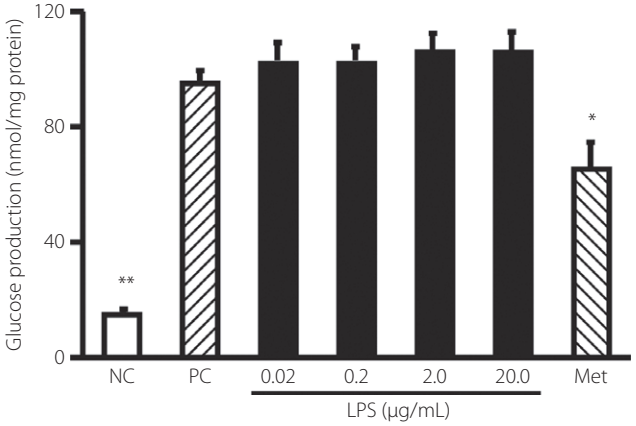

Figure 3 | No suppressive effects of lipopolysaccharide (LPS) on gluconeogenesis in primary hepatocytes. Glucose production was measured in primary hepatocytes treated with or without LPS (2 h after exposure to LPS concentrations ranging between 0.02 and 20.0 μg/mL). White bar, negative control without gluconeogenesis precursors (NC); black bar, LPS 0.02–20.0 μg/mL; right hatched bar, positive control (PC); left hatched bar, 5 mmol/L metformin (Met). Values are mean ± standard error ($n = 5$). *$P < 0.05$, **$P < 0.01$ vs positive control.

Effects of LPS on gluconeogenesis in perfused livers

The effects of LPS on gluconeogenesis from lactate were investigated in perfused rat livers. Glucose output into the perfusate was stabilized under 2 μmol/g/h. After the perfusate had been changed from basic buffer to Krebs–Ringer buffer containing 2 mmol/L lactate from 15 to 105 min, glucose output rapidly increased and reached a steady state at approximately 10 μmol/g/h. After the washout of lactate, glucose output decreased to 2 μmol/g/h. An intraperitoneal injection of LPS (0.5 mg/kg) 4 h before liver perfusion did not significantly change the glucose output from that in the control (Figure 4a). In Figure 4b, LPS (0.1–0.5 mg/kg/30 min) was infused continuously from 55 to 85 min with perfusion buffer, and had no significant effect on glucose output. Metformin (5 mmol/L) was infused continuously from 55 to 85 min with perfusion buffer, and significantly suppressed glucose output. These results suggest that the effects of LPS on glucose output in perfused rat livers were not significantly different from those in the controls.

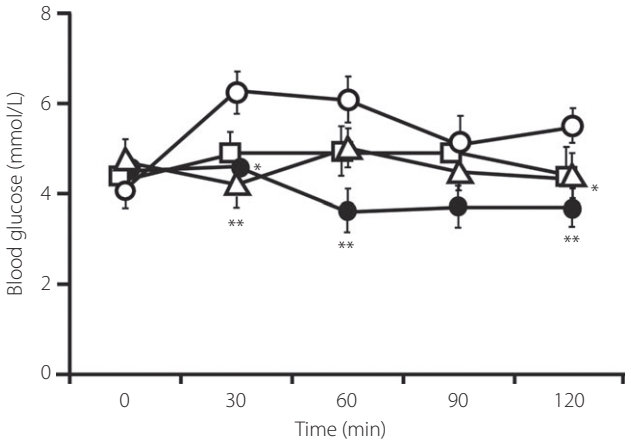

Figure 2 | Lipopolysaccharide (LPS) decreased gluconeogenesis from pyruvate. LPS (0.1–0.5 mg/kg bodyweight) or saline (control) was injected intraperitoneally to rats fasted for 16 h. After 4 h, blood glucose levels were measured, and pyruvate (1 g/kg bodyweight) was then injected intraperitoneally into the rats. Blood glucose levels during the pyruvate tolerance test were measured at intervals of 30 min after the pyruvate injection. White circles, control; white squares, negative control without pyruvate injection; white triangles, LPS (0.1 mg/kg bodyweight); black circles, LPS (0.5 mg/kg bodyweight). Values are means ± standard error ($n = 5$). *$P < 0.05$, **$P < 0.01$ significantly different from the value for the injection of saline intraperitoneally.

Time-course of the production of cytokines induced by LPS in splenocytes

As the suppressive effects of LPS on hepatic gluconeogenesis might be due to its indirect effects on the liver, we examined the production of cytokines in immunocytes with the addition of LPS. The main cytokines, NO, TNF-α, IFN-γ, IL-1β, IL-6 and IL-10, induced by LPS in splenocytes were measured. The exposure of splenocytes to LPS significantly increased the production of NO, TNF-α, IFN-γ, IL-1β and IL-10 in a concentration-dependent manner (Figure 5).

Figure 5 shows the time-course of increases in NOx, TNF-α, IFN-γ, IL-1β and IL-10 production after the application of LPS at concentrations of 0.01–1.0 μg/mL. IL-6 was undetectable in the absence and presence of LPS (0.01–1.0 μg/mL) at all sampling times (data not shown).

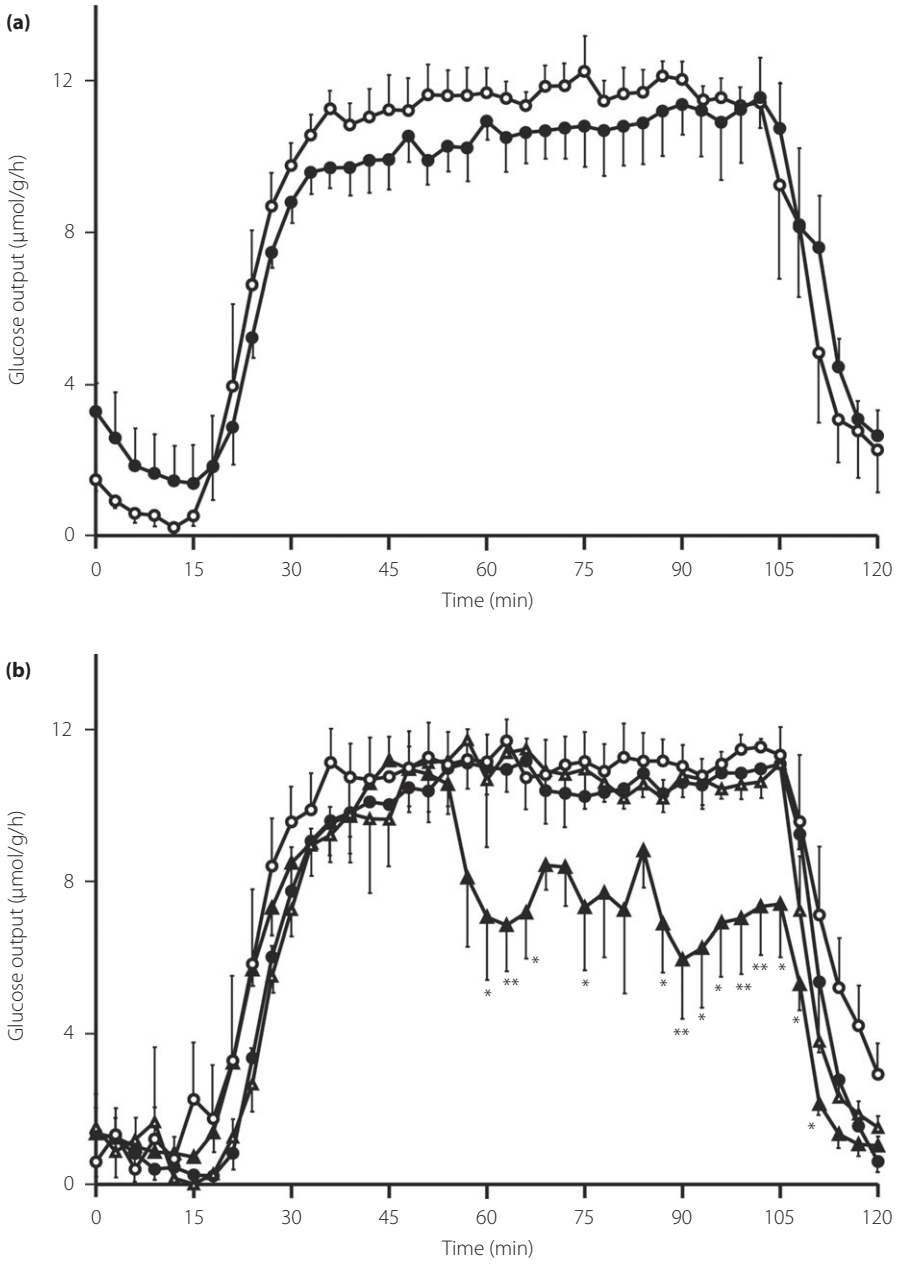

Figure 4 | No suppressive effects of lipopolysaccharide (LPS) on glucose output in perfused rat livers. (a) Four hours after the intraperitoneal injection of LPS, livers were perfused with Krebs–Ringer buffer, and from 15 min to 105 min with 2 mmol/L lactate. White circles, control; black circles, LPS. Values are mean ± standard error (n = 4). (b) LPS or vehicle was not injected intraperitoneally, and livers were perfused with Krebs–Ringer buffer. LPS (0.1 or 0.5 mg/kg bodyweight/30 min) or metformin (5 mmol/L) was administered in perfusion buffer from 55 min to 85 min. White circles, control; black circles, LPS (0.5 mg/kg bodyweight/30 min); white triangles, LPS (0.1 mg/kg bodyweight/30 min); black triangles, metformin (5 mmol/L). Values are mean ± standard error (n = 4). *P < 0.05, **P < 0.01 vs control.

Effects of cytokines on gluconeogenesis in hepatocytes

The effects of cytokines (NO, TNF-α, IFN-γ, IL-1β, IL-6 and IL-10), which are known to be induced by LPS, on gluconeogenesis in isolated hepatocytes were examined. After a 120-min incubation with cytokines, glucose concentrations were measured in order to investigate the effects of cytokines on gluconeogenesis in hepatocytes (Figure 6). The inclusion of SNP in incubation medium resulted in the inhibition of glucose synthesis without insulin. This inhibition was significant at concentrations >50 μmol/L SNP (Figure 6a). TNF-α, IFN-γ, IL-1β, IL-6 and IL-10 had no significant effects on gluconeogenesis in hepatocytes (Figure 6b–f). Glucose production was significantly reduced in the absence

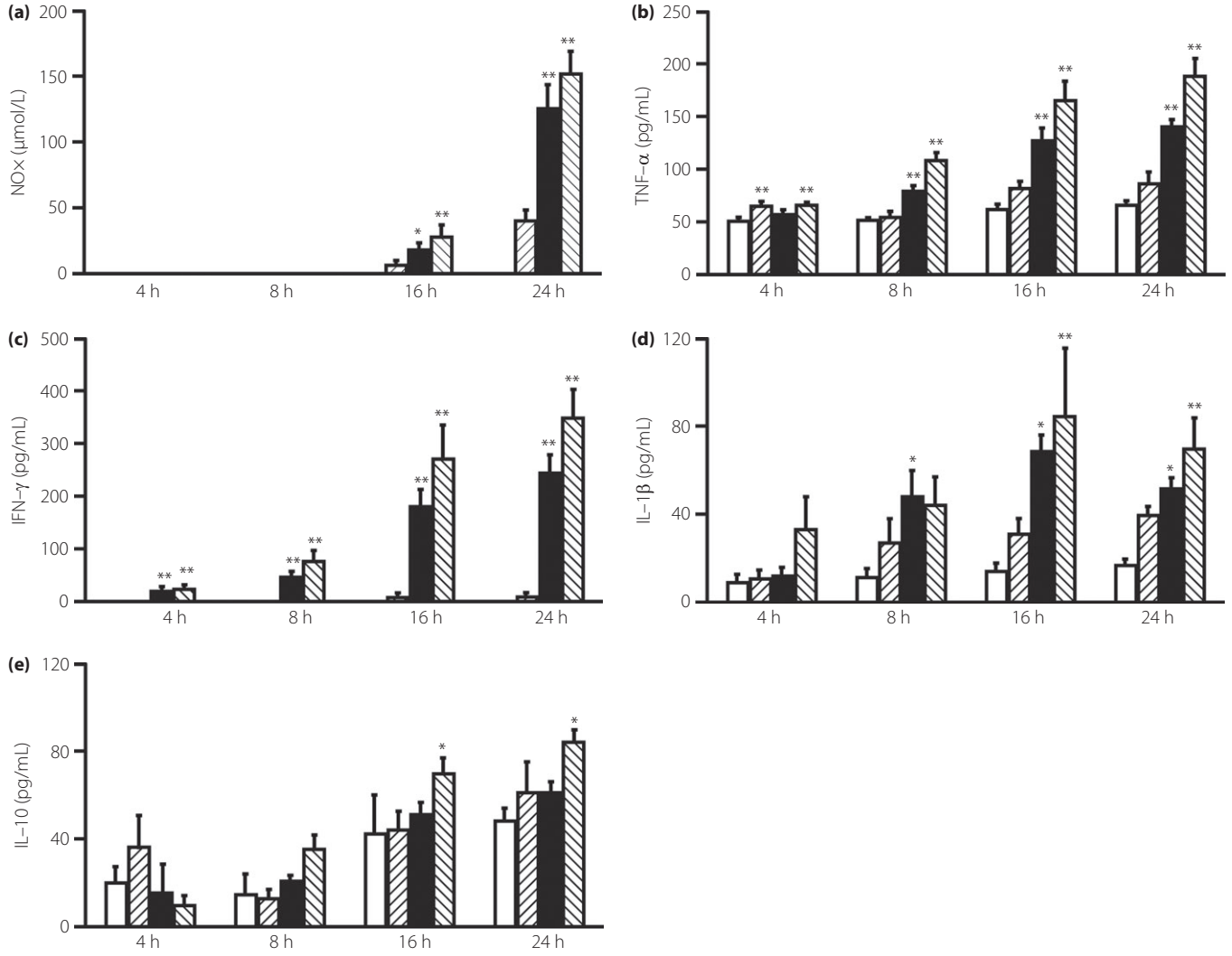

Figure 5 | Time-courses of the production of cytokines by splenocytes exposed to various concentrations of lipopolysaccharide (LPS). The concentrations of the LPS-induced cytokines, (a) nitrate + nitrite (NOx), (b) tumor necrosis factor-α (TNF-α), (c) interferon-γ (IFN-γ), (d) interleukin (IL)-1β, and (e) IL-10 in the culture medium of splenocytes were measured after 4, 8, 16 and 24 h. White bar, control; right hatched bar, LPS (0.01 μg/mL); black bar, LPS (0.1 μg/mL); left hatched bar, LPS (1.0 μg/mL). Values are mean ± standard error ($n = 6$). *$P < 0.05$, **$P < 0.01$ significantly different from the corresponding control.

of gluconeogenesis precursors in incubation medium or in the presence of 5 mmol/L metformin. These results show that LPS-induced NO is a key regulator of hepatic gluconeogenesis.

Effects of NO on adenosine monophosphate-activated protein kinase-α phosphorylation in hepatocytes

In order to investigate the underlying mechanisms by which NO suppresses gluconeogenesis, we examined the effects of NO and metformin on adenosine monophosphate-activated protein kinase-α (AMPKα) using isolated hepatocytes. The therapeutic effects of metformin are mediated by the activation of AMPK, leading to a decrease in hepatic gluconeogenesis. After a 2-h exposure, 50 μmol/L SNP and 5 mmol/L metformin stimulated the phosphorylation of Thr^{172} of AMPKα, suggesting the activation of AMPK (Figure 7).

Effects of LPS on co-cultured splenocytes and hepatocytes

The interaction between splenocytes and hepatocytes in response to LPS was investigated *in vitro*. After splenocytes and hepatocytes were co-cultured for 2 h in culture medium with/without LPS (1.0 μg/mL), the mRNA expression levels of PEPCK and G6Pase, master regulators of glucose production, were examined in hepatocytes. G6Pase mRNA expression levels were significantly lower in LPS-treated hepatocytes than in LPS-untreated hepatocytes, whereas those of PEPCK were lower in LPS-treated hepatocytes than in LPS-untreated hepatocytes (Figure 8).

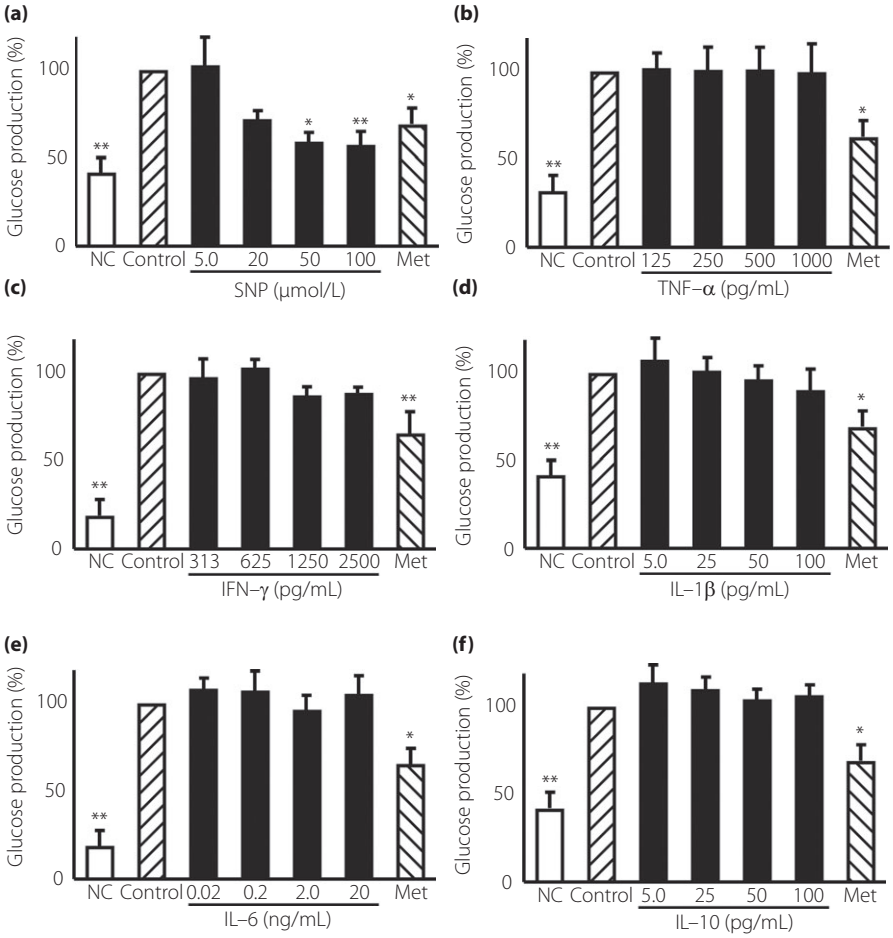

Figure 6 | Sodium nitroprusside (SNP) reduced glucose production in hepatocytes. Primary hepatocytes isolated from rats were incubated with various concentrations of (a) SNP, (b) tumor necrosis factor-α (TNF-α), c) interferon-γ (IFN-γ), (d) interleukin (IL)-1β, (e) IL-6 and (f) IL-10 for 2 h. White bar, negative control without gluconeogenesis precursors (NC); black bar, various concentrations of cytokines; right hatched bar, positive control (PC); left hatched bar, 5 mmol/L metformin (Met). Values are mean ± standard error (n = 5). *P < 0.05, **P < 0.01 vs positive control.

Time-course of blood NOx concentrations after the LPS injection

In order to investigate the *in vivo* effects of LPS on NO production, we examined the time-course of blood NOx concentrations after the LPS injection using rats. Blood NOx concentrations were significantly higher in the LPS group (0.5 mg/kg) than in the control group from 4 h to 24 h (Figure 9). NOx concentrations were slightly higher in the LPS group (0.1 mg/kg) than in the control group. NOx concentrations at 4 h were significantly lower in the presence of L-NMMA (10 mg/kg), which was intravenously injected 220 min after LPS (0.5 mg/kg i.p.), than in its absence. These results show that LPS significantly increased the *in vivo* production of NO, which could suppress hepatic gluconeogenesis.

Inhibitory effects of L-NMMA on LPS action in the pyruvate tolerance test

In the pyruvate tolerance test, the pretreatment of L-NMMA (10 mg/kg i.v.) significantly reduced the inhibitory effects of LPS (0.5 mg/kg i.p.) on gluconeogenesis 60 min after the pyruvate injection (pretreatment, 5.5 ± 0.23 mmol/L; no pretreatment, 3.6 ± 0.41 mmol/L, P < 0.05 vs pretreatment).

DISCUSSION

The results of the present study showed that LPS, a substance that produces endotoxic shock, decreased gluconeogenesis from pyruvate *in vivo*. We also showed that the LPS injection increased the plasma levels of TNF-α and IFN-γ, which are inflammatory cytokines. A previous study reported that TNF-α prevented enhancements in glucagon-stimulated gluconeogenesis from lactate in isolated hepatocytes[9]. In that study, hepatocytes were pre-incubated for 4 h in culture medium containing fetal calf serum and insulin. Hepatocytes were then cultured for 44 h in the absence of fetal calf serum, and stimulated with glucagon for an additional 4 h in medium M199 supplemented with TNF-α and [^{14}C]lactate. The rate of gluconeogenesis was then assessed radiochemically. However, another study reported that TNF-α stimulated gluconeogenesis by increasing plasma

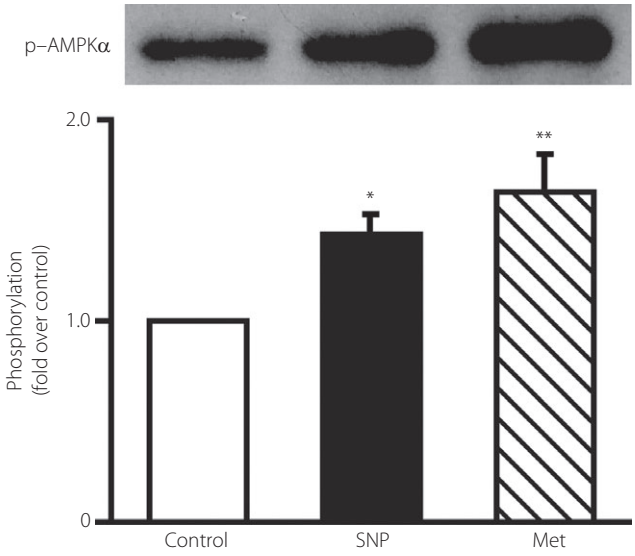

Figure 7 | Sodium nitroprusside (SNP) stimulated adenosine monophosphate-activated protein kinase-α (AMPKα) phosphorylation (p-AMPKα) in hepatocytes isolated from rats. After 2-h exposure, AMPKα phosphorylation was significantly stimulated by 50 μmol/L SNP or 5 mmol/L metformin (Met). White bar, control; black bar, 50 μmol/L SNP; left hatched bar, 5 mmol/L Met. Values are mean ± standard error (n = 5). *P < 0.05, **P < 0.01 vs control.

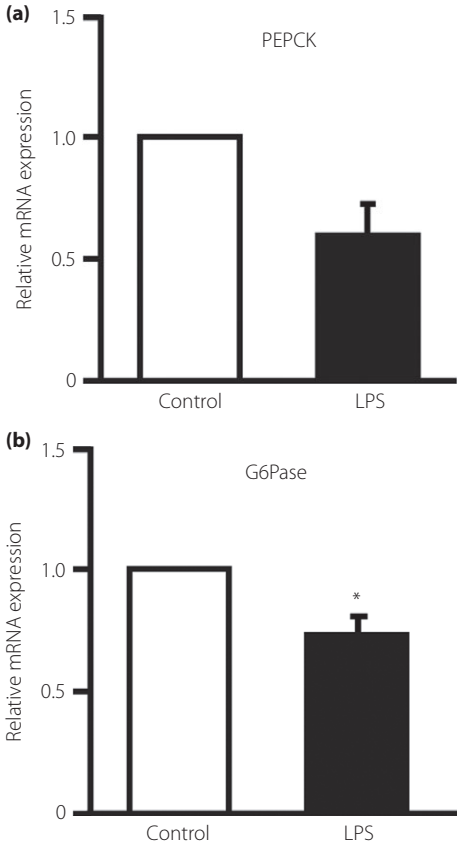

Figure 8 | Lipopolysaccharide (LPS) suppressed messenger ribonucleic acid (mRNA) expression levels of gluconeogenesis in hepatocytes co-cultured with splenocytes. After splenocytes and hepatocytes were co-cultured for 2 h in culture medium with/without LPS (1.0 μg/mL), the mRNA expression levels of (a) phosphoenolpyruvate carboxykinase (PEPCK) and (b) G6Pase were examined in hepatocytes. The mRNA expression levels of G6Pase were significantly lower in LPS-treated hepatocytes than in LPS-untreated hepatocytes, whereas those of PEPCK were lower in LPS-treated hepatocytes than in LPS-untreated hepatocytes. White bar, control; black bar, LPS (1.0 μg/mL). Values are mean ± SE (n = 5). *P < 0.05 vs the control.

glucagon levels *in vivo*[15]. In that study, rats were injected intraperitoneally with TNF-α, followed by collagenase digestion of the liver to isolate hepatocytes. Isolated hepatocytes were incubated for 2 h in culture medium containing alanine as a glucogenic amino acid, and the rate of hepatic gluconeogenesis was measured. The present results showed that TNF-α failed to affect gluconeogenesis in freshly isolated hepatocytes. In the present study, isolated hepatocytes were incubated for 2 h in Kreb–Ringer buffer with gluconeogenic precursors (lactate and pyruvate) in the presence of TNF-α. The reasons for the discrepancy between the present results and the findings of Christ *et al.* and Blumberg *et al.* remain unknown, but might be attributed to differences in experimental protocols and conditions.

Regarding IFN-γ, Wang *et al.*[16] reported that a deficiency in IFN-γ reduced hepatic G6Pase activity and enhanced glucose tolerance in mice. In that study, the bodyweight of and food intake by IFN-γ knockout mice were lower, whereas their physical activity was greater than the control. Nevertheless, the present results showed that the addition of IFN-γ to culture medium did not affect gluconeogenesis in isolated rat hepatocytes. The reason for the discrepancy in our results and the findings of Wang *et al.* remains unclear, but again might be ascribed to differences in the methods and species used.

Chronic inflammatory signaling is generally regarded as a key cause of insulin resistance[17,18]. We herein showed that the acute exposure of hepatocytes to TNF-α and IFN-γ did not have any effects on gluconeogenesis. Therefore, the acute and chronic effects of inflammatory cytokines appear to differ.

iNOS, known as an LPS-stimulated protein, has beneficial roles in host immunity[19]. iNOS mutant mice have an increased susceptibility to infection, showing the importance of iNOS in host defenses against infectious disease[20,21]. Excessive amounts of NO produced by iNOS might react with the superoxide anion to generate the peroxynitrite radical, which has detrimental effects on cells[22]. Regarding glucose metabolism, iNOS was shown to reduce glucose tolerance and increase insulin resistance[11,23]. Conversely, in septic shock, the genetic deletion of iNOS in mice maintained hepatic gluconeogenesis, leading to the prevention of hypoglycemia[24]. Therefore, the impact of iNOS on glucose metabolism remains controversial.

The disruption of endothelial NOS causes insulin resistance[25], whereas its activation ameliorates insulin resistance in

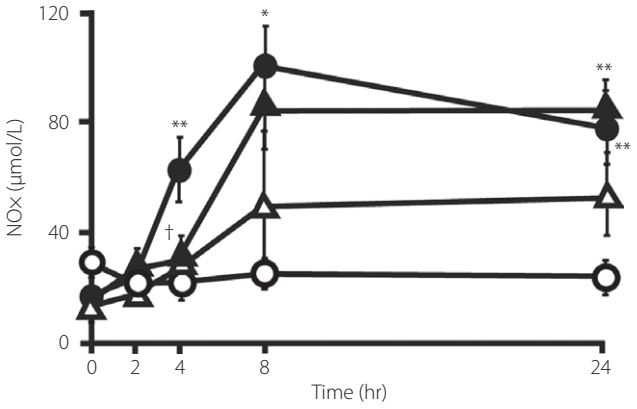

Figure 9 | Lipopolysaccharide (LPS) significantly increased the production of nitrate + nitrite (NOx), *in vivo*. Blood NOx concentrations were measured, followed by an intraperitoneal injection of saline (control) or LPS (0.1–0.5 mg/kg bodyweight). The levels of NOx produced were measured after 2, 4, 8 and 24 h. NOx concentrations at 4 h were significantly lower in the presence of N^G-monomethyl-L-arginine (L-NMMA; 10 mg/kg bodyweight), which was intravenously injected 220 min after LPS (0.5 mg/kg bodyweight i.p.), than in its absence. White circles, control; white triangles, LPS (0.1 mg/kg bodyweight); black circles, LPS (0.5 mg/kg bodyweight); black triangles, LPS (0.5 mg/kg bodyweight) in the presence of L-NMMA (10 mg/kg bodyweight). Values are mean ± standard error ($n = 5$). *$P < 0.05$, **$P < 0.01$ significantly different from the control. †$P < 0.05$ significantly different from the value for LPS (0.5 mg/kg bodyweight) in the absence of L-NMMA.

diabetic mice[26]. Therefore, endothelial NOS is currently attracting attention for its central role in the regulation of glucose metabolism. The production of an appropriate amount of NO might be important in the regulation of glucose metabolism and prevention of infection in patients with diabetes.

Endotoxic hypoglycemia has been reported in patients with liver cirrhosis and kidney failure[27,28], implying that a dysfunction in gluconeogenic organs is associated with septic hypoglycemia.

In the present study, after the LPS injection, gluconeogenesis from pyruvate *in vivo* decreased in the pyruvate tolerance test. Blood NOx concentrations also significantly increased 4 h after the LPS injection. Regarding our study *in vitro*, SNP suppressed gluconeogenesis in isolated hepatocytes, whereas LPS stimulated the production of NOx in isolated splenocytes. In co-cultures of splenocytes and hepatocytes, LPS suppressed G6Pase mRNA expression in hepatocytes. Collectively, these results suggest that NO produced by splenocytes is involved in hypoglycemia with endotoxic shock. LaNoue *et al.*[4] reported that the intraperitoneal administration of LPS to rats reduced G6Pase activity, which is consistent with the present study.

Regarding the LPS dosage applied in *in vivo* experiments, Raetzsch *et al.*[29] reported that the intraperitoneal application of LPS at dosages of 0.05–0.5 mg/kg reduced blood glucose levels in a dose-dependent manner, with the maximal response being observed at 0.5 mg/kg. In that study, the intraperitoneal application of LPS (5 mg/kg) was lethal. Consistent with their study, we used LPS dosages of 0.1–0.5 mg/kg to examine the effects of LPS on glycemia and the pyruvate tolerance test.

The spleen is a lymphatic organ that is located in the left hypochondriac region. Blood in this organ is rich in macrophages. The splenic vein directly connects to the portal vein. LPS is known to induce splenomegaly, which is associated with a larger number of immune cells than that in a healthy spleen. Thus, in endotoxin shock, NO produced by splenocytes is more likely to play an important role in the inhibition of hepatic gluconeogenesis.

The limitations of the present study need to be considered. In this study, we did not carry out experiments using a co-culture of hepatocytes and Kupffer cells, which have been shown to release NO by various stimuli including LPS[30,31]. NO produced by Kupffer cells in response to LPS might inhibit hepatic gluconeogenesis. Further research is warranted. Another limitation is that we carried out liver perfusion using the flow-through method, not the re-circulation method. In our perfusion method, some of the NO produced by Kupffer cells in response to LPS might have been washed out by fresh perfusion solution, thereby decreasing its effects on hepatocytes. Goto *et al.*[32] carried out liver perfusion using the re-circulation method in 10-day-old rats, and reported that the intraperitoneal pretreatment of LPS reduced hepatic gluconeogenesis. The reasons for the discrepancy between the present results and the findings of Goto *et al.* remain unknown, but might be attributed to differences in the experimental protocols used. In the present study, the suppression of glucose output was not observed in the liver perfusion experiment with LPS. Therefore, further studies are required.

Decreased NO levels in blood have been reported with diabetes in humans[33] and rats[34]. The regulation of NOS-mediated NO signaling might open a new avenue for the treatments for diabetes.

In conclusion, high blood concentrations of NO in endotoxemia might cause hypoglycemia through the inhibition of hepatic gluconeogenesis in an insulin-independent pathway. The regulation of iNOS-mediated NO signaling could lead to new treatments for hypoglycemia in endotoxemia.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. American College of Chest Physicians/Society of Critical Care. Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Crit Care Med* 1992; 20: 864–874.
2. Suzuki A, Uno M, Arima K, *et al.* A case report: sepsis associated with hypoglycemia. *J Jpn Assoc Infect Dis* 1994; 68: 986–989.
3. van der Crabben SN, Blümer RM, Stegenga ME, *et al.* Early endotoxemia increases peripheral and hepatic insulin sensitivity in healthy humans. *J Clin Endocrinol Metab* 2009; 94: 463–468.

4. LaNoue KF, Mason AD Jr, Daniels JP. The impairment of glucogenesis by gram negative infection. *Metabolism* 1968; 17: 606–611.
5. Filkins JP, Cornell RP. Depression of hepatic gluconeogenesis and the hypoglycemia of endotoxin shock. *Am J Physiol* 1974; 227: 778–781.
6. Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol* 2001; 1: 135–145.
7. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000; 406: 782–787.
8. Eisenstein AB, Strack I. Effects of glucagon on carbohydrate synthesis and enzyme activity in rat liver. *Endocrinology* 1968; 83: 1337–1348.
9. Christ B, Nath A. Impairment by interleukin1β and tumor necrosis factor α of the glucagon induced increase in phosphoenolpyruvate carboxykinase gene expression and gluconeogenesis in cultured rat hepatocytes. *Biochem J* 1996; 320: 161–166.
10. Feingold KR, Moser A, Shigenaga JK, *et al.* Inflammation inhibits the expression of phosphoenolpyruvate carboxykinase in liver and adipose tissue. *Innate Immun* 2012; 18: 231–240.
11. Sugita H, Kaneki M, Tokunaga E, *et al.* Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance. *Am J Physiol Endocrinol Metab* 2002; 282: E386–E394.
12. Horton RA, Ceppi ED, Knowles RG, *et al.* Inhibition of hepatic gluconeogenesis by nitric oxide: a comparison with endotoxic shock. *Biochem J* 1994; 299: 735–739.
13. Fujita Y, Hosokawa M, Fujimoto S, *et al.* Metformin suppresses hepatic gluconeogenesis and lowers fasting blood glucose levels through reactive nitrogen species in mice. *Diabetologia* 2010; 53: 1472–1481.
14. Yamada K, Hosokawa M, Fujimoto S, *et al.* Effect of corosolic acid on gluconeogenesis in rat liver. *Diabetes Res Clin Pract* 2008; 80: 48–55.
15. Blumberg D, Hochwald S, Burt M, *et al.* Tumor necrosis factor alpha stimulates gluconeogenesis from alanine *in vivo*. *J Surg Oncol* 1995; 59: 220–224.
16. Wong N, Fam BC, Cempako GR, *et al.* Deficiency in interferon-gamma results in reduced body weight and better glucose tolerance in mice. *Endocrinology* 2011; 152: 3690–3699.
17. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006; 444: 860–867.
18. Abdul-Ghani MA, DeFronzo RA. Pathogenesis of insulin resistance in skeletal muscle. *J Biomed Biotechnol* 2010; 2010; Article ID 476279.
19. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993; 329: 2002–2012.
20. MacMicking JD, Nathan C, Hom G, *et al.* Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 1995; 81: 641–650.
21. Wei XQ, Charles IG, Smith A, *et al.* Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 1995; 375: 408–411.
22. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007; 87: 315–424.
23. Shinozaki S, Choi CS, Shimizu N, *et al.* Liver-specific inducible nitric-oxide synthase expression is sufficient to cause hepatic insulin resistance and mild hyperglycemia in mice. *J Biol Chem* 2011; 286: 34959–34975.
24. Albuszies G, Vogt J, Wachter U, *et al.* The effect of iNOS deletion on hepatic gluconeogenesis in hyperdynamic murine septic shock. *Intensive Care Med* 2007; 33: 1094–1101.
25. Duplain H, Burcelin R, Sartori C, *et al.* Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. *Circulation* 2001; 104: 342–345.
26. Abudukadier A, Fujita Y, Obara A, *et al.* Tetrahydrobiopterin has a glucose-lowering effect by suppressing hepatic gluconeogenesis in an endothelial nitric oxide synthase-dependent manner in diabetic mice. *Diabetes* 2013; 62: 3033–3043.
27. Nouel O, Bernuau J, Rueff B, *et al.* A common complication of septicemia in cirrhosis. *Arch Intern Med* 1981; 141: 1477–1478.
28. Miller SI, Wallace RJ Jr, Musher DM, *et al.* Hypoglycemia as a manifestation of sepsis. *Am J Med* 1980; 68: 649–654.
29. Raetzsch CF, Brooks NL, Alderman JM, *et al.* Lipopolysaccharide inhibition of glucose production through the Toll-like receptor-4, myeloid differentiation factor 88, and nuclear factor kappa b pathway. *Hepatology* 2009; 50: 592–600.
30. Hsu CM, Wang JS, Liu CH, *et al.* Kupffer cells protect liver from ischemia-reperfusion injury by an inducible nitric oxide synthase-dependent mechanism. *Shock* 2002; 17: 280–285.
31. Billiar TR, Curran RD, Ferrari FK, *et al.* Kupffer cell:hepatocyte cocultures release nitric oxide in response to bacterial endotoxin. *J Surg Res* 1990; 48: 349–353.
32. Goto M, Zeller WP, Lichtenberg RC. Decreased gluconeogenesis and increased glucose disposal without hyperinsulinemia in 10-day-old rats with endotoxic shock. *Metabolism* 1994; 43: 1248–1254.
33. Vanizor B, Orem A, Karahan SC, *et al.* Decreased nitric oxide end-products and its relationship with high density lipoprotein and oxidative stress in people with type 2 diabetes without complications. *Diabetes Res Clin Pract* 2001; 54: 33–39.
34. Tang ST, Zhang Q, Tang HQ, *et al.* Effects of glucagon-like peptide-1 on advanced glycation endproduct-induced aortic endothelial dysfunction in streptozotocin-induced diabetic rats: possible roles of Rho kinase- and AMP kinase-mediated nuclear factor κB signaling pathways. *Endocrine* 2016; 53: 107–116.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 | Gluconeogenesis in perfused rat livers.
Appendix S2 | Immunoblotting analysis.
Table S1 | Primer sequences used in quantitative reverse transcription polymerase chain reaction